Ionic net fluxes through the *in situ* epithelia of larval *Caudiverbera caudiverbera* (Anura, Leptodactylidae)

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The nature of the *in vivo* epithelial exogenous Cl^- and Na^+ uptake mechanisms at gilled and gill-less stages of tadpoles of the Chilean frog *Caudiverbera caudiverbera* was studied.

Ion net fluxes (Jn) in tadpoles acclimated in tap-water (controls), deionized water and in choline.Cl and Na_2SO_4 solutions were measured.

Larvae took up both Cl^- and Na^+ from diluted NaCl solutions. Tadpoles kept in deionized water showed the highest fluxes; larvae from choline.Cl solutions showed elevated $JnNa^+$ while those from Na_2SO_4 had very high $JnCl^-$. The differences found in the Jn suggest that the ion uptake mechanisms might be partially independent.

Acclimation solutions slightly affected the plasma ion concentration values with respect to controls. Concentrations of Cl^- and Na^+ of animals from deionized water showed a decreasing tendency; the level of those ions was lower in plasma of tadpoles maintained in Na_2SO_4 and choline.Cl, respectively.

It was postulated that the *in vivo* ion uptake must be active.

INTRODUCTION

The mechanisms of water and ion equilibrium in amphibian larvae are not as extensively understood as in adults (see DUELLMAN & TRUEB, 1985). Several authors have summarized our knowledge on different aspects of the osmoregulation in larvae of these vertebrates (ALVARADO, 1979; WARBURG & ROSENBERG, 1990).

We attempted to improve our comprehension of the nature of the epithelial ionic uptake mechanisms during the larval ontogenetic cycle of *Caudiverbera caudiverbera*. It is a large aquatic leptodactylid endemic to Chile (CEI, 1962; DIAZ, 1983; SALIBIÁN, 1974,

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1980), characterized by the fact that after metamorphosis juveniles remain in contact with water throughout their lives. Only few anurans are known to share this unusual type of life cycle with *C. caudiverbera*.

The sequence of morphological events during metamorphosis of this species is similar to those described in the larval development of terrestrial anurans. ZAMORANO et al. (1988) studied the pattern of urinary nitrogen excretion along their larval-juvenile transition; they found a physiological peculiarity: in spite of their permanent aquatic condition, a shift from ammoniotelism to ureotelism occurs as in the water-to-land model of metamorphosis.

Our main purpose was to study the characteristics of exogenous Cl^- and Na^+ uptake mechanisms through the intact epithelia of prometamorphic and climactic larval stages of *C. caudiverbera* after acclimations in solutions of different chemical composition.

MATERIALS AND METHODS

PREPARATION OF THE ANIMALS

Larvae were collected in natural ponds at Melipilla, Chile. The animals were held in the laboratory at 18°C in aquaria containing tap-water renewed daily and fed *ad libitum* with cooked spinach. The photoperiod was LD 12/12 It has been demonstrated that the ion transport mechanisms in amphibian larvae are affected by environmental factors such as temperature (PARSONS, 1975) and season (COX & ALVARADO, 1979). Since the experiments reported here were conducted at constant temperature and photoperiod and carried out during a short period of time, it is reasonable to conclude that our results were not irregularly affected by those factors.

Before the experiments, animals were divided into four groups and left without food for 15-20 days in one of the following solutions: (1) tap-water (control group); (2) deionized water (prepared from distilled water passed through a Kotterman 7002 resin column); (3) 3.4 $mEq.l^{-1}$ choline.Cl; (4) 3.4 $mEq.l^{-1}$ Na_2SO_4. Animals tolerated acclimation conditions without signs of perturbation. In previous studies carried out on adults of *C. caudiverbera* (MORENO et al., 1978; SALIBIÁN, 1970, 1973), we have demonstrated that animals kept in tap-water could be considered as controls since the evaluated morphological and chemical parameters were identical to those of frogs maintained in NaCl dilute solutions.

The Cl^- and Na^+ concentrations in the tap-water were periodically checked during the acclimation period; they were (means $\pm$ SEM, in $mEq.l^{-1}$) 3.2 ± 0.5 ($n = 12$) and 2.9 ± 0.4 ($n = 12$), respectively.

Animals were staged adapting the notation of GOSNER (1960). The considered stages were the following: (1) gilled (or ETKIN's prometamorphic) stages: 36-37 (toes partially or totally separated) and 38-39 (metatarsal tubercle and pigment free patches on the inner surface of toes appear); and (2) gill-less (or climactic) stages: 42-43 (incomplete regression of the tail) and 44-46 (complete resorption of the tail).

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The body weight range (in grams) of tadpoles at stages 36-37 was 18.6-24.0, and 34.2-40.4 at stages 38-39, in the gill-less larvae the ranges were 28.9-34.0 (stages 42-43) and 19.5-21.3 (stages 44-46).

MEASUREMENT OF IONIC NET FLUXES

Animals were weighed in water with a digital Sartorius 2250 balance at the beginning and at the end of the experiments; no statistically significant changes were observed between these two data sets of values. A polyethylene PE 20 cannula was inserted into the cloaca of the larvae and tightly fastened by a concentric subepithelial ligature; by this means renal and intestinal contamination of the external bath was avoided.

Each cannulated tadpole was individually placed in a container with 200 ml of its acclimation solution and maintained in that condition for about 16 h; thus physiological alterations due to the manipulation stress were avoided, as it was found in our laboratory (unpublished). Then the external solution was carefully replaced by siphoning with 150-200 ml of 1.7 $mEq.l^{-1}$ NaCl solutions. Aliquots of the external bath for subsequent analyses of Cl^- and Na^+ concentrations were taken at the beginning of the experiments and every 30-45 mn during the following 6-7 h.

The Cl^- and Na^+ concentrations in those samples were plotted against time and the net fluxes calculated from the slopes of the regression lines For this calculation a mean volume of the external bath was considered. The experiments were performed in spring; each tadpole was used in only one experiment. Animals that showed abnormally high negative net fluxes during the first hour of the experiment or with signs of skin damage were discarded.

Since our flux data corresponded to animals fasted for 2-3 weeks prior to the measurements, food must be discarded as an additional source for balancing the Cl^- and Na^+ losses ALVARADO & MOODY (1970) have shown that even in the fasting condition, the gut cannot be a physiologically relevant site for ion accumulation from the external bath because the volume drunk by the tadpoles can be considered negligible.

BLOOD SAMPLING

Blood samples were drawn from animals anesthetized in 0.3 % tricaine (Sigma) solutions through a little cut in the extreme of the ventricle. Blood was collected in glass tubes with heparin (Biochimie) at 5 $IU.ml^{-1}$ of blood. Plasma was separated by centrifugation at 4°C in a Sorvall RC-2B centrifuge at 600 g for 15-20 min, and diluted 1/100 before the analyses.

ANALYTICAL TECHNIQUES

Chloride concentration was evaluated potentiometrically (SANDERSON, 1952) with a Radiometer titration unit (pHmeter 26, TTT auto titrator and autoburette type ABU-1C) or with a Buchler digital H-2500 chloridometer. Sodium concentration was determined by emission photometry with an Eppendorf flame photometer.

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Expression of the results and statistical analyses

All data are given as means ± SEM (standard error of the mean). Net ion fluxes are expressed as $\mu Eq.h^{-1}.100\ g^{-1}$ animal weight. Plasma ion concentrations are in $mEq.l^{-1}$.

Student's t test was used to test differences between means of ion net fluxes. Results of plasma Cl^- and Na^+ concentrations of tap-water, deionized water and choline.Cl animals were tested for normality (Kolmogorov-Smirnov); then a two-way analysis of variance followed by Scheffé-Dunnett tests for comparison of means were made. Plasma ion concentrations of Na_2SO_4 larvae only were tested for significant differences between 36-37 and 44-46 stages data of animals by means of Student's t test.

Results

Net ionic fluxes in control tap-water acclimated larvae (Table I)

The magnitude of Cl and Na^+ epithelial net fluxes (Jn) were similar at stages 36-37 and 42-43; in the latter the fluxes were reduced by 50 %. At the end of metamorphosis (stages 44-46), there was a further reduction and a partial dissociation of the fluxes, with $JnCl^-$ being significantly higher than $JnNa^+$; this dissociation was not observed at the two earlier stages

Net ionic fluxes in deionized water acclimated larvae (Table II)

When compared with those of controls from tap-water, the absolute $JnCl^-$ and $JnNa^+$ values in these larvae were always very high. At early stages (36-37 and 38-39), $JnNa^+$ was higher than $JnCl^-$ while the opposite occurred at the last gill-less stages of the metamorphosis.

Net ionic fluxes in choline Cl solutions acclimated larvae (Table III)

In all studied stages the incubation of animals in Na-free solutions resulted in higher $JnNa^+$ than $JnCl^-$. The differences between net fluxes remained unchanged during stages with gills, but increased abruptly later as metamorphosis advanced. It is interesting to note that in these animals the $JnCl^-$ were comparable to those of control tap-water tadpoles of the same stage.

Net ionic fluxes in Na_2SO_4 solutions acclimated larvae (Table IV)

Results show that at stage 37 both $JnCl^-$ and $JnNa^+$ were low and similar. When animals reached stages 44-46 fluxes were dissociated, with $JnCl^-$ being seven times higher than $JnNa^+$ which, in turn, was almost identical to that found in control animals of the same age

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Table I. - Cl^- and Na^+ net fluxes (Jn) through the *in vivo* epithelia of larval *Caudiverbera caudiverbera* acclimated in tap-water. Fluxes were measured from 1.7 mEq l^{-1} NaCl solutions; data in $\mu Eq.h^{-1}.100\ g^{-1}$ body mass (means ± SEM); N: number of experiments.

Larval stages	N	$JnCl^-$	$JnNa^+$	$JnCl^- - JnNa^+$ [1]	p [2]
36 - 37	15	+17.6 ± 1.3	+18.0 ± 1.9	–0.4 ± 0.1	N.S.
42 - 43	15	+9.9 ± 0.9	+10.9 ± 0.8	–1.0 ± 0.1	N.S.
44 - 46	15	+3.6 ± 0.5	+3.0 ± 0.3	+0.6 ± 0.1	< 0.025

1. Mean differences of paired data ± SEM.
2. Statistical significance of the differences.

Table II. - Cl^- and Na^+ net fluxes (Jn) through the *in vivo* epithelia of larval *Caudiverbera caudiverbera* acclimated in deionized water. Fluxes were measured from 1.7 mEq.l^{-1} NaCl solutions; data in $\mu Eq.h^{-1}.100\ g^{-1}$ body mass (means ± SEM); N: number of experiments.

Larval stages	N	$JnCl^-$	$JnNa^+$	$JnCl^- - JnNa^+$ [1]	p [2]
36 - 37	15	+42.7 ± 4.2	+71.4 ± 3.2	–28.7 ± 0.9	< 0.001
38 - 39	15	+23.3 ± 2.5	+32.9 ± 2.3	–9.6 ± 0.5	< 0.025
42 - 43	17	+23.1 ± 1.9	+21.8 ± 2.1	+1.3 ± 1.0	N.S.
44 - 46	17	+57.3 ± 1.6	+29.5 ± 1.1	+27.8 ± 0.9	< 0.001

1. Mean differences of paired data ± SEM.
2. Statistical significance of the differences.

Table III. - Cl^- and Na^+ net fluxes (Jn) through the *in vivo* epithelia of larval *Caudiverbera caudiverbera* acclimated in 3.4 mEq.l^{-1} choline Cl solutions. Fluxes were measured from 1.7 mEq.l^{-1} NaCl solutions; data in $\mu Eq.h^{-1}.100\ g^{-1}$ body mass (means ± SEM); N: number of experiments.

Larval stages	N	$JnCl^-$	$JnNa^+$	$JnCl^- - JnNa^+$ [1]	p [2]
36 - 37	8	+20.2 ± 2.0	+28.9 ± 1.5	–8.7 ± 2.3	< 0 01
38 - 39	10	+21.7 ± 0.7	+29.4 ± 2.8	–7.7 ± 2.5	< 0.025
42 - 43	8	+2.3 ± 0.4	+14.9 ± 1.6	–12.6 ± 0.9	< 0.001
44 - 46	13	+6.1 ± 1.4	+27.0 ± 2.3	–20.9 ± 1.0	< 0.001

1. Mean differences of paired data ± SEM.
2. Statistical significance of the differences.

Source MNHN, Paris

Table IV. - Cl^- and Na^+ net fluxes (Jn) through the *in vivo* epithelia of larval *Caudiverbera caudiverbera* acclimated in 3.4 mEq.l^{-1} Na_2SO_4 solutions. Fluxes were measured from 1.7 mEq.l^{-1} NaCl solutions; data in µEq.h^{-1}.100 g^{-1} body mass (means ± SEM); N: number of experiments.

Larval stages	N	$JnCl^-$	$JnNa^+$	$JnCl^- - JnNa^+$ [1]	p [2]
37	7	+2.9 ± 0.2	+2.8 ± 0.3	+0.1 ± 0.1	N.S.
44 - 46	7	+19.7 ± 0.7	+2.7 ± 0.4	+17.0 ± 0.1	< 0.001

1. Mean differences of paired data ± SEM.
2. Statistical significance of the differences.

Table V. - Plasma chloride and sodium concentration of *Caudiverbera caudiverbera* larvae acclimated in different solutions. Data in mEq.l^{-1} (means ± SEM); in parenthesis, number of animals; N.M.: not measured.

Ion	Larval stages	Acclimation solutions			
		Tap-water	Deionized water	Choline.Cl (3.4 mEq l^{-1})	Na_2SO_4 (3.4 mEq.l^{-1})
Cl^-	36-37	68.5 ± 1.6 (10)	56.4 ± 3.2 (10)[2]	71.6 ± 1.7 (10)	63.0 ± 1.6 (7)
	38-39	69.3 ± 1.8 (10)	63.8 ± 2.0 (10)	75.6 ± 2.4 (10)	N.M.
	42-43	71.1 ± 0.4 (13)	74.2 ± 1.7 (10)[1]	70.9 ± 2.0 (8)	N.M.
	44-46	75.4 ± 1.1 (10)[1]	69.3 ± 0.3 (11)[1]	75.4 ± 2.1 (10)	66.3 ± 2.2 (7)
Na^+	36-37	79.6 ± 3.0 (10)	72.6 ± 5.6 (10)	76.2 ± 1.6 (8)	87.3 ± 3.5 (7)
	38-39	82.2 ± 2.6 (10)	67.5 ± 1.7 (10)[2]	78.1 ± 2.4 (10)	N.M.
	42-43	96.8 ± 2.7 (13)[1]	87.3 ± 1.2 (10)[1]	86.5 ± 2.5 (8)[1]	N.M.
	44-46	106.3 ± 2.4 (10)[1]	97.9 ± 2.5 (11)[1]	95.2 ± 1.8 (10)[1]	105.6 ± 2.6 (7)[3]

1. Within each acclimation solution, significantly different from the 36-37 stage larvae ($p < 0.05$).
2. Within each larval stage range, significantly different from the tap-water larvae ($p < 0.05$)
3. Significantly different from the 36-37 stage larvae ($p < 0.001$).

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PLASMA Cl^- AND Na^+ CONCENTRATIONS (TABLE V)

In comparison to the Cl^- concentrations in plasma of control larvae, there was a trend towards its reduction in animals from deionized water and Cl-free solutions. The Cl^- concentration in plasma of larvae kept in choline.Cl solutions remained unaltered with respect to tap-water controls.

When compared with control tap-water acclimated tadpoles, the Na^+ plasma concentration of animals from deionized water and Na-free solutions showed a tendency to decrease; the plasma Na^+ concentration of Na_2SO_4 acclimated animals was not different from those of controls.

In all groups of animals the plasma Na^+ concentrations showed a significant increase in the gill-less stages whereas the Cl^- concentrations did exhibit a similar but less pronounced trend

DISCUSSION

Both gilled and gill-less tadpoles of *Caudiverbera caudiverbera* showed the capacity to take up Cl^- and Na^+ from dilute external NaCl solutions. This ability was found to exist independently of the age of the animals and of the acclimation conditions.

Our preparation does not allow to discriminate precisely the sites where transport occurs at each stage. However, evidence reported by several authors (see ALVARADO, 1979) indicates that during anuran metamorphosis there are gill-to-skin shifts as site for ion translocations. In tadpoles of *C. caudiverbera* indirect evidence in favor of the postulated shift along the larval-juvenile transition from the gill as the dominant organ towards the skin as the main site for ion transport was reported by GONZALEZ et al (1979) They measured the specific activity of ouabain sensitive ($Na^+ + K^+$)-ATPase in isolated gills and skin of larvae at different stages of animals stored in tap-water. At stages 30-35 they showed the existence of enzymatic activity only in gills; at the intermediate stages 36-39 the activity was found both in gills and skin in the proportion of 1/3, later, close to the end of metamorphosis (stages 40-43), most of the enzyme activity was detected on the skin.

Relative to the NaCl solutions used in flux measurements, plasma of *C caudiverbera* tadpoles were always markedly hyperionic with respect to Cl^- and Na^+; the ratios of their concentrations in plasma to external bathing media varied between 40 and 62.

We conclude that our data allow us to postulate that the ion accumulation processes of larval *C. caudiverbera* are active and must be attributed to epithelial mechanisms located, according to the age of the animals, in gills and/or skin.

The acclimation condition affects the magnitude of ionic net fluxes when animals are transferred to NaCl solutions. In control tap-water animals (Table I), the $JnCl^-$ and the $JnNa^+$ were practically similar; this could be interpreted as an indication of a linked uptake of Cl^- and Na^+. However, at climactic stages a clear-cut difference among the net fluxes appeared, suggesting the existence of a differentiation in their ion uptake

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mechanisms, the animals being able to take up a fraction of the exogenous Cl^- by a Na^+-independent ionic exchange system.

After keeping tadpoles in deionized water (Table II), the ion net fluxes were augmented at least three times with respect to controls; the increases were constant and not dependent on the developmental stages. Similar response to ion-free incubation of the animals was shown in intact *R. catesbeiana* tadpoles (ALVARADO & MOODY, 1970); in the same species, BROONKOOM & ALVARADO (1971) demonstrated an increase in the (Na^+ + K^+)-ATPase activity in gill homogenate of "salt depleted" animals.

Tadpoles coming from Na-free solutions (Table III) took up Na^+ at higher rate than Cl^-, independently of the considered age. Conversely, the $JnCl^-$ in animals from Cl-free solutions (Table IV) were higher than the $JnNa^+$; the ion net fluxes were among the lowest registered in the early stages In both groups the differences between fluxes were greatly enhanced in climactic stages.

It must be emphasized that the observed differences in epithelial net ion flux values between the controls and the remaining three groups cannot be attributed to changes in the plasma Cl^- and Na^+ levels (see Table V) It might be necessary to search for that correlation in the intracellular condition of the epithelial cells involved in ion transport processes rather than in that of the extracellular fluids (COX & ALVARADO, 1983; LARSEN, 1988) The relative constancy found in the Cl^- and Na^+ concentrations of the extracellular compartment may explain the excellent tolerance of these animals to incubation for 2-3 weeks in artificial environmental conditions.

Tadpoles in ion-free media are exposed to a severe limitation in their capacity to compensate for the normal ion and water losses We postulate that the particular condition of these animals may trigger an endocrine compensatory reponse, increasing the circulatory levels of hormones involved in the hydromineral balance regulation through epithelia, especially neurohypophysial peptides and aldosterone. The integrated action of these hormones upon their effectors may act by promoting an increased reabsorption of water and ions, principally Na^+, thus reducing the losses and, consequently, maintaining the plasma levels stable over the ion deprived incubation period of time. The existence of such compensatory responses was shown in adults of several anuran species (BENTLEY, 1969; CRABBE, 1963; ROJAS et al., 1987) and in embryos of *Bufo arenarum* (CASTAÑÉ et al., 1987).

Finally, the magnitude of the *in vivo* ion net fluxes of control tadpoles tend to decrease as metamorphosis goes on and become close to those measured in adults (GARCIA ROMEU et al., 1969). This fact might be the consequence of the reduction in the ion exchange areas due to resorption of the gills and tail and/or ontogenetic selective modifications in the magnitude of the unidirectional fluxes.

RESUMEN

Se estudió la naturaleza de los mecanismos epiteliales de captación *in vivo* de Cl^- y Na^+ exógenos en estadios larvales branquiados y no branquiados de larvas de la rana chilena *Caudiverbera caudiverbera.*

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Se midieron los flujos iónicos netos (Jn) en larvas aclimatadas en agua potable (controles), agua deionizada y en soluciones de Cl.colina y SO_4Na_2.

Los animales captaron Cl^- y Na^+ desde soluciones diluidas de ClNa. Los Jn más altos se observaron en larvas de agua deionizada; las de Cl.colina tuvieron los $JnNa^+$ elevados mientras que los $JnCl^-$ fueron mayores en las de SO_4Na_2. Las diferencias halladas en los Jn sugieren que los mecanismos de captación iónica podrían ser parcialmente independientes.

Las soluciones de preadaptación afectaron ligeramente las concentraciones plasmáticas de Cl^- y de Na^+ con respecto a las de los controles. Las de los animales de agua deionizada mostraron una tendencia a la reducción; los niveles de esos iones resultaron disminuídos en larvas mantenidas respectivamente en SO_4Na_2 y Cl.colina.

Se postuló que el transporte de iones *in vivo* debe ser de carácter activo.

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